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(54) A method of forming a macromolecular microgene polymer

(57) A method of forming a macromolecular microgene polymer comprises allowing DNA polymerase to act on oligonucleotides A and B complementary at least partially to each other to effect polymerase chain reaction. According to the present invention, there can be obtained a polymer consisting of a repeating microgene, which is efficiently and simply formed.

FIG. 1a

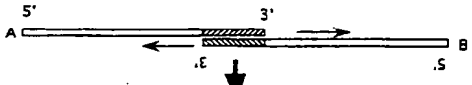
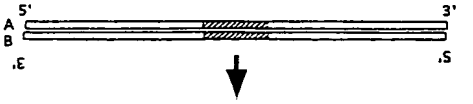


FIG. 1b



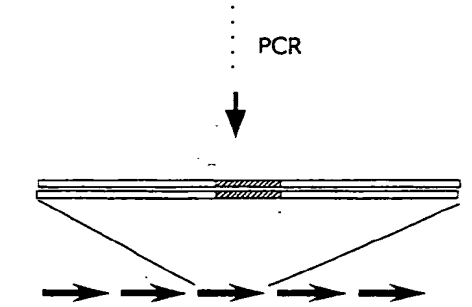


FIG. 1c

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Description

FIELD OF THE INVENTION

The present invention relates to a method of forming a macromolecular microgene polymer by use of DNA polymerase.

BACKGROUND OF THE INVENTION

The advent of evolutional molecular engineering has made it feasible to create an enzyme (protein) forming the basis of life reaction or a gene coding therefor in laboratories. By this technology, an enzyme (protein) with new activity not occurring in nature can be produced and expected for use in various applications to the fields of medicine and engineering.

An enzyme (protein) or a gene coding therefor is composed of a polymer of amino acids or nucleotides as a block unit. In evolutional molecular engineering, a molecule with desired activity is selected from a pool of polymers consisting of random amino acid or nucleotide block units.

However, even if it is attempted to prepare polymers with every combination, there is a limit to the physical amount of compounds which can be synthesized, so there is a limit to the number of blocks which can be linked, and as a consequence, a too large protein or gene cannot be created. Further, in consideration of an in vitro evolutional system for translating a protein from a nucleic acid polymer, the appearance of "termination codon" terminating the translation is a great problem. Therefore, a microgene which is large to a certain extent is preferably used as a block unit to form a gene coding for a large protein.

There is the hypothesis that a large gene was born by repeatedly polymerizing a small gene (microgene) (S. Ohno & J. T. Epplen, Proc. Natl. Acad. Sci. U.S.A. 80:3391-3395). Because it is considered that a polypeptide rich in a simple repeating structure can easily have a stable secondary structure, evolutional molecular engineering directed at large proteins or genes requires the techniques of repeatedly polymerizing a short structural unit to synthesize a macromolecule (Nature 367:323-324, 1994).

At present, a rolling circle synthesis method is reported as a method of preparing a polymer consisting of a short repeating DNA unit (PNAS 92:4641-4645, 1995).

However, this method should go through a plurality of steps including phosphorylation reaction, linkage reaction, polymerization reaction, double-stranded chain forming reaction, so its complicated reaction system is problematic.

Under these circumstances, there have been demands for developments in a reaction system in which a gene polymer can be formed more simply.

SUMMARY OF THE INVENTION

The object of the present invention is to provide a method of efficiently and simply forming a polymer consisting of a repeating microgene.

As a result of their extensive research, the present inventors found that a macromolecular microgene polymer can be formed efficiently and simply by allowing DNA polymerase to act on oligonucleotides complementary at least partially to each other, to complete the present invention.

That is, the present invention is a method of forming a macromolecular microgene polymer, which comprises allowing DNA polymerase to act on oligonucleotides A and B complementary at least partially to each other to effect polymerase chain reaction (PCR).

The DNA polymerase includes exonucleases, particularly those acting in the 3'→5' direction. In addition, the DNA polymerase is preferably thermally stable.

In the method of forming a macromolecular microgene polymer according to the present invention, the 3-terminals of oligonucleotide A and/or oligonucleotide B can contain at least one nucleotide not capable of forming a base pair with the other oligonucleotide.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 a to FIG. 1 c is a schematic drawing showing the method of the present invention.
FIG. 2 is a photograph showing the result of agarose gel electrophoresis.
FIG. 3 shows genes synthesized by the method of the present invention.
FIG. 4 is a photograph showing the result of agarose gel electrophoresis.
FIG. 5 is a photograph showing the result of agarose gel electrophoresis.
FIG. 6 is a photograph showing the result of agarose gel electrophoresis.
FIG. 7 is a photograph showing the result of agarose gel electrophoresis.

FIG. 8 is a photograph showing the result of agarose gel electrophoresis.

FIG. 9 shows genes synthesized by the method of the present invention.

FIG. 10 is a photograph showing the result of SDS polyacrylamide gel electrophoresis.

DETAILED DESCRIPTION OF THE INVENTION

Hereinafter, the present invention is described in detail.

As shown in FIG. 1a, two oligonucleotides (oligonucleotides A and B) with complementary regions being at least partially to each other are synthesized before conducting polymerase chain reaction according to the present invention. In the present invention, oligonucleotides A and B are synthesized so as to be complementary to each other particularly in their 3'-terminal sequences. The number of oligonucleotides forming a complementary chain to each other is preferably at least 6, more preferably at least 8, although there is no particular limitation.

Alternatively, oligonucleotides A and B may be synthesized such that the 3-terminals of oligonucleotide A and/or oligonucleotide B contain 1 or more nucleotides (preferably 1 to 3 nucleotides) being not capable of forming base pairs with the other oligonucleotide. By this operation, the efficiency of reaction can be raised.

Further, because one object of the present invention is to create a completely new gene polymer not occurring in the nature, said 2 oligonucleotides are not particularly limited and may be selected arbitrarily insofar as they are at least partially complementary to each other. The synthesized oligonucleotides form a double-stranded chain in only the part of their complementary region.

As used herein, the term "complementary" can refer not only to the relationship between adenine and thymidine or guanine and cytosine, but also to the relationship between guanine and thymine or the like insofar as oligonucleotides A and B are at least partially complementary to each other.

Oligonucleotides A and B function as primers to initiate PCR at their complementary region (a double-stranded chain in FIG. 1a), where the single-stranded chain (i.e. not forming the double-stranded chain with oligonucleotide B) of oligonucleotide A acts as a template for synthesizing oligonucleotide B and the single-stranded chain (i.e. not forming the double-stranded chain with oligonucleotide A) of oligonucleotide B acts as a template for synthesizing oligonucleotide A (FIG. 1a). If PCR is conducted by allowing e.g. thermostable DNA polymerase with the 3'→5' exonuclease activity to act on said 2 oligonucleotides, double-stranded DNA is synthesized as a repeating unit (FIG. 1b). By further continuing the PCR, large DNA consisting of continuous repeating units is synthesized (FIG. 1c).

The PCR using polymerase (e.g. Taq polymerase) is carried out by conducting 1 cycle at 94 °C for 10 to 120 seconds, 30 to 65 cycles each at 69°C for 10 to 120 seconds, and 1 cycle at 69 °C for 3 to 7 minutes.

To conduct PCR efficiently, additional reaction at 94 °C for 10 minutes and at 69 °C for 10 minutes is preferably carried out before conducting the above cycles.

In this manner, the complementary part of the 2 oligonucleotides serves as a self-primer and the other oligonucleotide serves as a template for synthesizing them, resulting in polymerization of DNA with double-stranded chain DNA as a repeating unit (FIG. 1b) with an extremely large number of copies in the same direction (FIG. 1c). In the present invention, the replacement, insertion and/or deletion of several nucleotides may occur between repeating units insofar as the repeating units form a complementary chain.

EXAMPLES

Hereinafter, the present invention is described in more detail by reference to Examples which however are not intended to limit the scope of the present invention.

Example 1

KY-794 (SEQ ID NO:1) and KY-795 (SEQ ID NO:2) were synthesized respectively as oligonucleotides A and B for use in PCR. The synthesized oligonucleotides A and B were composed of 22 and 23 nucleotides respectively where their 3'-terminal 8 nucleotides were complementary to each other (the sequence at the 15- to 22-positions in KY-794 was complementary to the sequence at the 15- to 22-positions in KY-795). Adenine (A) was added to the 3'-terminal of KY-795 to prevent formation of a base pair with KY-794.

The conditions for PCR using the above oligonucleotides in a 50 μl reaction volume are as follows:

KY-794 (SEQ ID NO:1)	20 pmol
KY-795 (SEQ ID NO:2)	20 pmol
dNTP	350 μM
$MgCl_2$	1.75 mM
Tris-HCl, pH 9.2	50 mM
$(NH_4)_2SO_4$	14 mM
Taq polymerase	2.6 units/50 μl

The Taq polymerase used was a mixture of Taq polymerase and Pwo polymerase contained in Expand™ Long Template PCR system (Boehringer).

PCR was carried out using 9600 or 2400 PCR system (Perkin Elmer) for cycle reaction under the following conditions:

94°C 10 minutes

69°C 10 minutes

(94 °C for 10 seconds and 69°C for 60 seconds) × 45 cycles

69°C 7 minutes

The enzyme was added when the system reached 94 °C.

The PCR product obtained under these conditions was subjected to 1.2 % agarose gel electrophoresis.

The result is shown in FIG. 2.

As can be seen from FIG. 2, DNA reaching several kilo base pairs or more can be polymerized in this method.

The polymer thus obtained was cloned into plasmid vector pTZ19R (Mead et al., Protein Eng.1:67-74 (1986)). For 4 clones (pSA32, pSA33, pYT5 and pYT8), their insert fragments were sequenced using a sequencer (Perkin Elmer).

The results are shown in FIG. 3. The nucleotide sequences determined for the respective clones are shown in SEQ ID NO: 3 for pSA32, SEQ ID NO: 4 for pSA33, SEQ ID NO: 5 for pYT5, and SEQ ID NO: 6 for pYT8.

In SEQ ID NO:3, the sequences at the 1- to 36-positions, the 40- to 75-positions and the 77- to 112-positions are identical with one another, so it is understood that a polymer was synthesized in which many of double-stranded chains as repeating units each consisting of 37 base pairs derived from KY-794 and KY-795 had been linked in the same direction. This applies to SEQ ID NOS:4-6.

In FIG. 3, "△" indicates the absence of the corresponding nucleotide in the linking region of the repeating units each consisting of the sequence derived from the oligonucleotides, and the underlined nucleotides are an insert of unknown origin in the linking region of the repeating units.

Example 2

In the reaction shown in Example 1 (FIG. 2), the 3'-terminal of KY795 had one nucleotide being not capable of forming a base pair with KY-794. In this example, polymerization was carried out using the combination of oligonucleotide KY-783 (SEQ ID NO:7) and oligonucleotide KY-794, i.e. the combination not forming such a mismatch.

The conditions for PCR were identical to those in Example 1. The PCR product obtained under these conditions was subjected to 1.2 % agarose gel electrophoresis.

The result is shown in FIG. 4.

As can be seen from FIG. 4, the efficiency of polymerization is improved when at least one nucleotide being not capable of forming a base pair with the other oligonucleotide is present at the 3'-terminal of the oligonucleotide (FIG. 4, lane 2).

Example 3

As shown in FIG. 5, KY-794 and KY-795 have a complementary region of 8 bases. In this example, polymerization

was carried out using oligonucleotide KY-845 (SEQ ID NO:8) and oligonucleotide KY-846 (SEQ ID NO:9) whose complementary region consisted of 6 nucleotides which is shorter by 2 bases than above. The composition of the reaction solution was the same as in Example 2 except that PCR was carried out under the following cycle conditions 1 or 2:

(Conditions 1)

94°C 10 minutes
63°C 10 minutes

(94 °C for 10 seconds and 63°C for 60 seconds) × 45 cycles

63°C 7 minutes;

(Conditions 2)

94°C 10 minutes
66°C 10 minutes

(94 °C for 10 seconds and 66°C for 60 seconds) × 45 cycles

66°C 7 minutes

The PCR products obtained under these conditions were subjected to 1.2 % agarose electrophoresis.

The results are shown in FIG. 5.

In FIG. 5, lanes 2 and 3 were obtained under Conditions 1 and lanes 4 and 5 under Conditions 2.

As can be seen from lanes 1 and 2 in FIG. 5, the polymerization reaction proceeds by decreasing the annealing temperature of the PCR cycle to 63°C even by the combination of the oligonucleotides having a complementary region of as short as 6 bases.

Example 4

In this example, thermostable DNA polymerase having the 3'→5' exonuclease activity was used as an enzyme for PCR. The 3'→5' exonuclease activity is important for raising polymerization efficiency. Accordingly, the importance of the 3'→5' exonuclease activity was examined using thermostable DNA polymerase lacking in the 3'→5' exonuclease activity.

The oligonucleotides used were KY-794 (SEQ ID NO:1) and KY-785 (SEQ ID NO:2). The PCR reaction solution had the same composition as in Example 1 except that the enzyme was 1.9 units/50 μl of thermostable polymerase Pfu DNA polymerase commercially available from Stratagene or Exo-Pfu DNA polymerase assumed to lack the 3'→5' exonuclease activity. PCR was carried out under the same cycleconditions as in Example 1. The PCR product obtained under these conditions was subjected to 2 % agarose gel electrophoresis.

The result is shown in FIG. 6.

As can be seen from FIG. 6, polymerization efficiency was dropped where Exo-Pfu DNA polymerase assumed to lack the 3'→5' exonuclease activity was used(lane 3) as compared with the case where Pfu DNA polymerase having the 3'→5'exonuclease activity was used (lane 2).

Example 5

In this example, polymerization was carried out using oligonucleotides with various sequences.

As shown in FIG. 7, the combination of KY-794 (SEQ ID NO:1) and KY-795 (SEQ ID NO:2) and the combination of KY-808 (SEQ ID NO:10) and KY-809 (SEQ ID NO:11) are identical in the number (= 8) of nucleotides forming a complementary chain, but are greatly different in the nucleotide composition of the complementary region.

KY-827 (SEQ ID NO:12), KY-828 (SEQ ID NO:13), KY-829 (SEQ ID NO:14) and KY-830 (SEQ ID NO:15) are partially modified sequences of KY-794 (SEQ ID NO:1), and KY-831 (SEQ ID NO:16), KY-832 (SEQ ID NO:17), KY-833 (SEQ ID NO:18), KY-834 (SEQ ID NO:19) and KY-835 (SEQ ID NO:20) are partially modified sequences of KY-795 (SEQ ID NO:2).

PCR was carried out under the same conditions as in Example 1 by using each of the following combinations: KY-794 (SEQ ID NO:1) and KY-795 (SEQ ID NO:2); KY-808 (SEQ ID NO:10) and KY-809 (SEQ ID NO:11); KY-827 (SEQ ID NO:12) and KY-795 (SEQ ID NO:2); KY-828 (SEQ ID NO:13) and KY-795 (SEQ ID NO:2); KY-829 (SEQ ID NO:14) and KY-795 (SEQ ID NO:2); KY-830 (SEQ ID NO:15) and KY-795 (SEQ ID NO:2); KY-794 (SEQ ID NO:1) and KY-831

(SEQ ID NO:16); KY-794 (SEQ ID NO:1) and KY-832 (SEQ ID NO:17); KY-794 (SEQ ID NO:1) and KY-833 (SEQ ID NO:18); KY-794 (SEQ ID NO:1) and KY-834 (SEQ ID NO:19); and KY-794 (SEQ ID NO:1) and KY-835 (SEQ ID NO:20). The PCR products obtained under these conditions were subjected to 2 % agarose gel electrophoresis.

The results are shown in FIGS. 7 and 8.

As can be seen from FIGS. 7 and 8, there are differences in efficiency but the polymerization reaction proceeds in any of the combinations of oligonucleotide sequences used.

Example 6

In order to allow the sequence of the resulting polymer to have diversity, polymerization was carried out using a partially randomized oligonucleotide. KY-812 (SEQ ID NO:21) and KY-795 (SEQ ID NO:2) were used as as primers. KY-812 (SEQ ID NO:21) is an oligonucleotide synthesized such that A, T, G or C is located at the 3- and 11-positions. The PCR reaction was carried out in the same manner as in Example 1. After the reaction, the resulting polymer was cloned into plasmid vector pTZ19R. For 4 clones (pYT15, pYT16, pYT20 and pYT21), their insert fragments were sequenced.

The results are shown in Table 9. The nucleotide sequences determined for the respective clones are shown in SEQ ID NO:22 for pYT15, SEQ ID NO:23 for pYT16, SEQ ID NO:24 for pYT20 and SEQ ID NO:25 for pYT22.

As can be seen from FIG. 9, the base at the 3-position had a preference for C, while A, T, G or C appeared as the base at the 11-position, so diversity was given to the sequence of the polymer.

Example 7

The protein encoded by the resulting polymer can be expressed in E. coli. The polymer obtained by the combination of KY-794 (SEQ ID NO:1) and KY-795 (SEQ ID NO:2) and the polymer obtained by the combination of KY-812 (SEQ ID NO:21) and KY-795 (SEQ ID NO:2) were cloned respectively into expression vector pET23b to give recombinants pYT32 and pYT33. The proteins derived from the polymers encoded by pYT32 and pYT33 were expressed in E. coli BL21 (DE3) and their cell extract was analyzed by SDS polyacrylamide gel electrophoresis on 15-25 % gradient gel.

The results are shown in FIG. 10. The molecular markers are of 97,400, 66,267, 42,400, 30,000, 20,100 and 14,000.

As can be seen from FIG. 10, proteins with a molecular weight of about 16 kDa derived from the polymers are expressed.

As illustrated above, a polymer consisting of a repeating microgene can be formed efficiently and simply according to the present invention.

SEQUENCE LISTING

SEQ ID NO: 1

LENGTH: 22

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACGGTCACC TGCACAAAGG CG 22

SEQ ID NO: 2

LENGTH: 23

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

CGGGATCCAC TGCACGCCTT TGA 23

SEQ ID NO: 3

LENGTH: 185

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCCC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG 185

SEQ ID NO: 4

LENGTH: 162

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGGCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGT

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCA

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCGCCA

GACGGTCAC 162

SEQ ID NO: 5

LENGTH: 280

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGT

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAA 280

SEQ ID NO: 6

LENGTH: 246

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACGGTCACCTGCACAAAGGCGTGCAGTAGATCCCGCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCG 646

SEQ ID NO: 7

LENGTH: 22

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

CGGGATCCAC TGCACGCCTT TG 22

SEQ ID NO: 8

LENGTH: 20

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACGGTCACC TGCACAGGCG 20

SEQ ID NO: 9

LENGTH: 21

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

CGGGATCCAC TGCACGCCTG A 21

SEQ ID NO: 10

LENGTH: 22

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACGGACACC TGCAAACGGA GC 22

SEQ ID NO: 11

LENGTH: 23

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

CGGGATCCAC TGCAGCTCCG TTA 23

SEQ ID NO: 12

LENGTH: 22

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

CTGGGTCACC TGCACAAAGG CG 22

SEQ ID NO: 13

LENGTH: 22

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACCCACACC TGCACAAAGG CG 22

SEQ ID NO: 14

LENGTH: 22

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACGGTGTGC TGCACAAAGG CG 22

SEQ ID NO: 15

LENGTH: 22

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACGGTCACG ACCACAAAGG CG 22

SEQ ID NO: 16

LENGTH: 23

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

CGGGATCCAC TCGTCGCCTT TGA 23

SEQ ID NO: 17

LENGTH: 23

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

CGGGATCCTG AGCACGCCTT TGA 23

SEQ ID NO: 18

LENGTH: 23

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

CGGGAAGGAC TGCACGCCTT TGA 23

SEQ ID NO: 19

LENGTH: 23

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

CGCCTTCCAC TGCACGCCTT TGA 23

SEQ ID NO: 20

LENGTH: 23

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GCGGATCCAC TGCACGCCTT TGA 23

SEQ ID NO: 21

LENGTH: 22

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GANGGTCACC NGCACAAAGG CG 22

SEQ ID NO: 22

LENGTH: 314

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACGGTCGCCGGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCCGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GATGGTCACCAGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCCGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCAGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGG 314

SEQ ID NO: 23

LENGTH: 408

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCAGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCCGCACAAAGGCGTGCAGTGGATCCCG

GATGGTCACCGGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCGT

GAGGGTCACCTGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCAC 408

SEQ ID NO: 24

LENGTH: 674

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GGTCACCGGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCAGCACAAAGGCGTGCAGTGGATCCC

GAAGGTCACCCGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCGCCGG

GAAGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCG

GAAGGTCACCGGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCAGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCCGCACAAAGGCGTGCAGTGGATCCCGCCGG

GATGGTCACCGGCAC 674

SEQ ID NO: 25

LENGTH: 373

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE:

GAGGGTCACCCGCACAAAGGCGTGCACTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCATTGGATCCCGCCGG

GACGGTCACCGGCACAAAGGGGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCC

GATGGTCACCCGCACAAAGGCGTGCAGTGGATCCC

GATGGTCACCCGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GAAGGTCACCGGCACAAAGGCGTGCAGTGGATCCCG 373

Claims

1. A method of forming a macromolecular microgene polymer, which comprises allowing DNA polymerase to act on oligonucleotides A and B complementary at least partially to each other to effect polymerase chain reaction.

2. The method of forming a macromolecular microgene polymer according to claim 1, wherein the DNA polymerase contains an exonuclease acting in the 3'→5' direction.

3. The method of forming a macromolecular microgene polymer according to claim 1, wherein the DNA polymerase is thermally stable.

4. The method of forming a macromolecular microgene polymer according to claim 1, wherein the 3-terminals of oligonucleotide A and/or oligonucleotide B contain at least one nucleotide not capable of forming a base pair with the other oligonucleotide.

FIG.1a

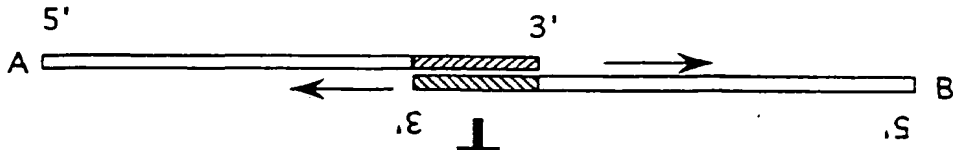
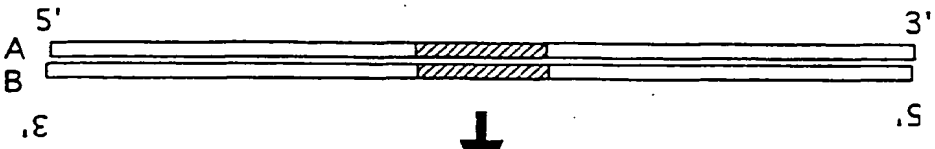


FIG.1b



PCR

FIG.1c

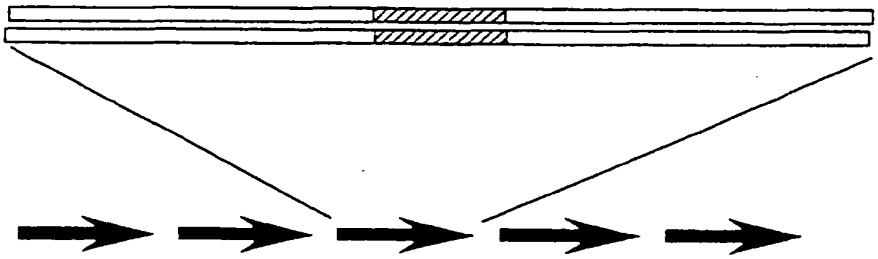


FIG.2

794 GACGGTCACCTGCACAAAGGCG

795 AGTTTCCGCACGTCACCTAGGGC

1. Size Marker
2. KY-794 & KY-795

FIG.3

GACGGTCACCTGCACAAAGGCG KY-794

AGTTTCCGCACGTCACCTAGGGC KY-795

(pSA32)

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCCC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCC△△

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

(pSA33)

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGGCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGT

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCA

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCGCCA

GACGGTCAC

(pYT5)

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGT

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC△

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC△

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAA

(pYT8)

GACGGTCACCTGCACAAAGGCGTGCAGTAGATCCCGCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGC

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCC△

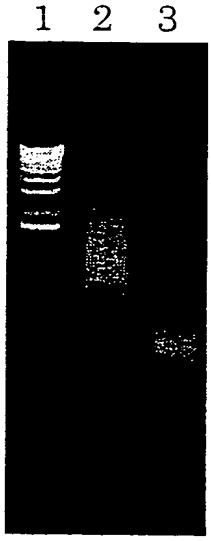
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG

GACGGTCACCTGCACAAAGGCG

FIG.4

794 GACGGTCACCTGCACAAAGGCG
795 AGTTTCCGCACGTCACCTAGGGC

794 GACGGTCACCTGCACAAAGGCG
783 GTTTCCGCACGTCACCTAGGGC

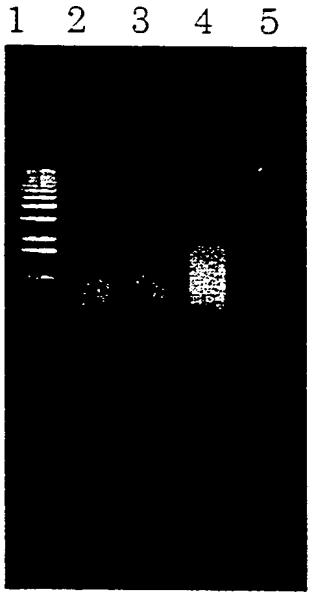


1. Size Marker
2. KY-794 & KY-795
3. KY-794 & KY-783

FIG.5

794 GACGGTCACCTGCACAAAGGCG
795 AGTTTCCGCACGTCACCTAGGGC

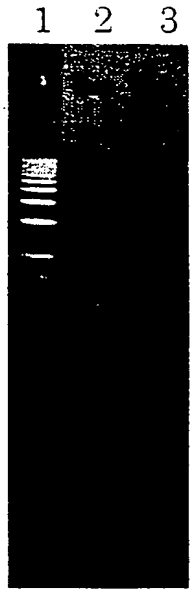
845 GACGGTCACCTGCACAGGCG
846 AGTCCGCACGTCACCTAGGGC



1. Size Marker
2. KY-794 & KY-795 63℃
3. KY-845 & KY-846 63℃
4. KY-794 & KY-795 66℃
5. KY-845 & KY-846 66℃

FIG.6

794 GACGGTCACCTGCACAAAGGCG
795 AGTTTCCGCACGTCACCTAGGGC

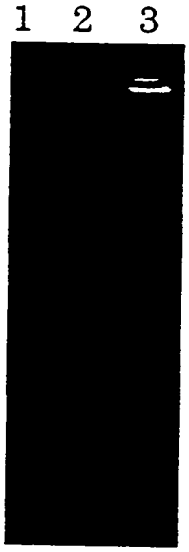


1. Size Marker
2. *Pfu* DNA Polymerase
3. Exo *Pfu* DNA Polymerase

FIG.7

794 GACGGTCACCTGCACAAAGGCG
795 AGTTTCCGCACGTCACCTAGGGC

808 GACGGTCACCTGCAAACGGAGC
809 ATTGCCTCGACGTCACCTAGGGC

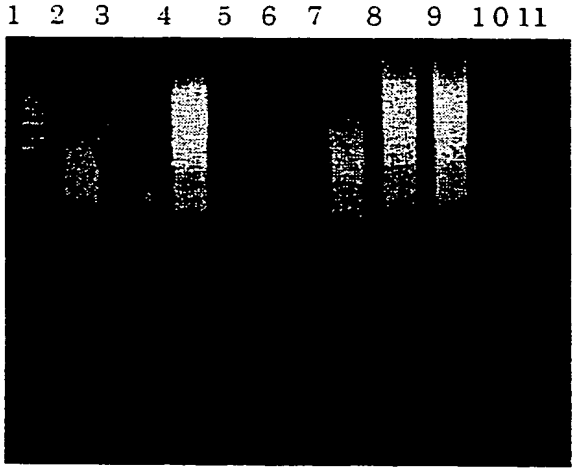


1. Size Marker
2. KY-794 & KY-795
3. KY-808 & KY-809

FIG.8

794 GACGGTCACCTGCACAAAGGCG
827 CTGGGTCACCTGCACAAAGGCG
828 GACCCACACCTGCACAAAGGCG
829 GACGGTGTGCTGCACAAAGGCG
830 GACGGTCACGACCACAAAGGCG

795 AGTTTCCGCACGTCACCTAGGGC
831 AGTTTCCGCTGCTCACCTAGGGC
832 AGTTTCCGCACGAGTCCTAGGGC
833 AGTTTCCGCACGTCAGGAAGGGC
834 AGTTTCCGCACGTCACCTTCCGC
835 AGTTTCCGCACGTCACCTAGGCG



1. Size Marker
2. KY-794 & KY-795
3. KY-827 & KY-795
4. KY-828 & KY-795
5. KY-829 & KY-795
6. KY-830 & KY-795
7. KY-794 & KY-831
8. KY-794 & KY-832
9. KY-794 & KY-833
10. KY-794 & KY-834
11. KY-794 & KY-835

FIG.9

GANGGTCACCNGCACAAAGGCG KY-812

AGTTTCCGCACGTCACCTAGGGC KY-795

(N = A, T, G, C)

(pYT15)

GACGGTCGCCGGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCCGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG
GATGGTCACCAGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCCGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCAGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCTGCACAAAGGCGTGCAGG

(pYT16)

GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCAGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCCGCACAAAGGCGTGCAGTGGATCCCG
GATGGTCACCGGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCGCGT
GAGGGTCACCTGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCGCCGG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCAC

(pYT20)

GGTCACCGGCACAAAGGCGTGCAGTGGATCCCGCCGG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCAGCACAAAGGCGTGCAGTGGATCCCΔ
GAAGGTCACCCGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCGCCGG
GAAGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCG
GAAGGTCACCGGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCAGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCCGCACAAAGGCGTGCAGTGGATCCCGCCGG
GATGGTCACCGGCAC

(pYT22)

GAGGGTCACCCGCACAAAGGCGTGCACTGGATCCCGCCGG
GACGGTCACCTGCACAAAGGCGTGCATTGGATCCCGCCGG
GACGGTCACCGGCACAAAGGGGTGCAGTGGATCCCG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCGCCGG
GACGGTCACCGGCACAAAGGCGTGCAGTGGATCCCΔ
GATGGTCACCCGCACAAAGGCGTGCAGTGGATCCCΔ
GATGGTCACCCGCACAAAGGCGTGCAGTGGATCCCGCCGG
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCΔ
GACGGTCACCTGCACAAAGGCGTGCAGTGGATCCCG
GAAGGTCACCGGCACAAAGGCGTGCAGTGGATCCCG

FIG.10

1. Molecular Weight Marker
2. pTZ19R/BL21(DE3)
3. pYT32/BL21(DE3)
4. pYT33/BL21(DE3)

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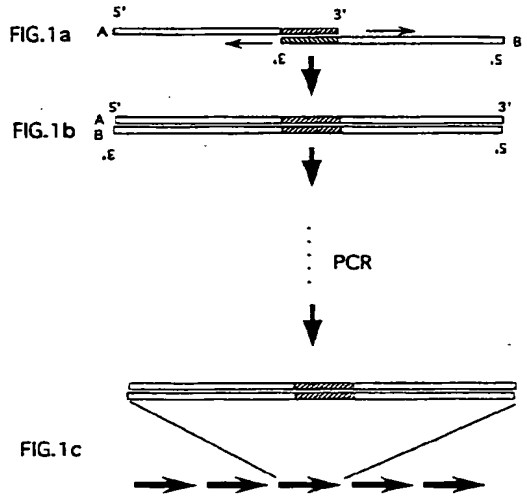
(54) A method of forming a macromolecular microgene polymer

(57) A method of forming a macromolecular microgene polymer comprises allowing DNA polymerase to act on oligonucleotides A and B complementary at least partially to each other to effect polymerase chain reaction. According to the present invention, there can be obtained a polymer consisting of a repeating microgene, which is efficiently and simply formed.



EP 0 812 911 A3

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EUROPEAN SEARCH REPORT

Application Number
EP 97 10 9308

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X Y	WO 93 09245 A (UNIV PITTSBURGH) 13 May 1993 (1993-05-13) * page 18, line 26 - line 34; claims 1-30; figures 1-4 *	1-3 2	C12N15/10 C12Q1/68
X	WHITE M J ET AL: "CONCATEMER CHAIN REACTION: A TAQ DNA POLYMERASE-MEDIATED MECHANISM FOR GENERATING LONG TANDEMLY REPETITIVE DNA SEQUENCES" ANALYTICAL BIOCHEMISTRY,US,ACADEMIC PRESS, SAN DIEGO, CA, vol. 199, no. 2, 1 December 1991 (1991-12-01), pages 184-190, XP000236491 ISSN: 0003-2697	1,3	
Y	* the whole document *	2	
X Y	WO 96 05296 A (INST MOLEKULARE BIOTECHNOLOGIE ;GREULICH KARL OTTO (DE); CELEDA DI) 22 February 1996 (1996-02-22) * claims 1-18; figures 1-8 *	1,3 2	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
Y	EP 0 669 401 A (HOFFMANN LA ROCHE) 30 August 1995 (1995-08-30) * claims 1-10 *	2	C12N C12Q
A	WO 92 01813 A (SYNGENE INC) 6 February 1992 (1992-02-06) * the whole document *		

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	21 February 2001	Hornig, H

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EUROPEAN SEARCH REPORT

Application Number
EP 97 10 9308

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,A	FIRE ANDREW ET AL: "Rolling replication of short DNA circles." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 92, no. 10, 1995, pages 4641-4645, XP002160947 1995 ISSN: 0027-8424 * the whole document *		
P,X	SHIBA KIYOTAKA ET AL: "Creation of libraries with long ORFs by polymerization of a microgene." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 94, no. 8, April 1997 (1997-04), pages 3805-3810, XP002160948 1997 ISSN: 0027-8424 * the whole document *	1-4	
P,X	WO 97 16546 A (GENENCOR INT) 9 May 1997 (1997-05-09) * claims 1-38; figures 1-6 *	1,3	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
P,X	WO 96 33207 A (GLAXO GROUP LTD ;LIPSHUTZ ROBERT J (US); STEMMER WILLEM P C (US)) 24 October 1996 (1996-10-24) * claims 1-18; figures 1-12 *	1-3	
P,A	EP 0 736 609 A (BOEHRINGER MANNHEIM GMBH) 9 October 1996 (1996-10-09) * claims 1-16 *		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	21 February 2001	Hornig, H

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21-02-2001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9309245 A	13-05-1993	EP 0656065 A	07-06-1995
		JP 7500734 T	26-01-1995
		US 5683872 A	04-11-1997
WO 9605296 A	22-02-1996	DE 4428651 C	29-02-1996
		AU 3160495 A	07-03-1996
EP 0669401 A	30-08-1995	US 5512462 A	30-04-1996
		CA 2143229 A	26-08-1995
		JP 8038198 A	13-02-1996
WO 9201813 A	06-02-1992	AU 649066 B	12-05-1994
		AU 8417391 A	18-02-1992
		EP 0542874 A	26-05-1993
		JP 6500014 T	06-01-1994
		NO 930235 A	22-01-1993
WO 9716546 A	09-05-1997	US 5756316 A	26-05-1998
		AU 727758 B	21-12-2000
		AU 7602396 A	22-05-1997
		CA 2236448 A	09-05-1997
		JP 11514526 T	14-12-1999
WO 9633207 A	24-10-1996	US 5834252 A	10-11-1998
		AU 5850996 A	07-11-1996
		EP 0824542 A	25-02-1998
		US 5837458 A	17-11-1998
		US 5928905 A	27-07-1999
EP 0736609 A	09-10-1996	EP 0736608 A	09-10-1996
		EP 0745687 A	04-12-1996
		JP 2928992 B	03-08-1999
		JP 9019299 A	21-01-1997

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